MOLECULAR FLUORESCENCE AND PHOTOSYNTHESIS

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The reality of chlorophyll forms with 10—12 nm half widths obtained by Gaussian analysis of the red absorption band of living plants is discussed. The main difficulties of the resolution and the problems of reality are the following:

1. Since the Gaussian components are very near to each other, the resolution of the band is more or less arbitrary.
2. Though the location of the components is clearer at low temperatures, specific low-temperature forms, not existing at higher temperatures may appear.
3. The true shape of the band is *not* Gaussian.
4. Since the bands of different chlorophyll forms are very near, the overlapping vibrational bands may introduce errors in the analysis.
5. The half widths of the red bands for solutions are without exception between 16 and 23 mm, far above the 10—12 nm suggested for several living forms.

On the basis of the fluorescence data, it is suggested that two or three main forms and several minor (low concentration) forms with reasonable band widths are to be accepted.

Micellar detergent (sodium-lauryl-sulphate) solutions of thionine, methylene blue, rhodamine 6G, and their mixtures, are studied as models of the photosynthetic unit. The absorption of light and the fluorescence of these dyes yield information on the presence of monomeric, aggregated and detergent complex forms of the dyes, and on the transfer of electronic excitation energy in these complex systems. The optimum average distance (24—40 Å) and the optimum number (10—20) of dye molecules within a micelle for efficient transfer were found.

True photosynthetic pigments (chlorophyll-a, chlorophyll-b and lutein) can be incorporated into triton-X100 micelles. The transfer of energy from chlorophyll-b to chlorophyll-a and from lutein to chlorophyll-a in these micellar solutions is discussed.

Introduction

Fluorescence and photosynthesis in plants are primarily due to the presence of plastid pigments. The energy of light absorbed at a given site of the photosynthetic unit is transferred over the ordered system of pigments to the reaction center. Here the energy may drive the primary photochemical reaction of photosynthesis or may migrate to a pigment molecule, being emitted in the form of fluorescence. Consequently, fluorescence and photosynthesis are competing processes. Inhibition or promotion of photosynthesis leads to an increase or decrease of the intensity of fluorescence emitted by *in vivo* chlorophylls. In addition, the absorption and fluorescence of pigment molecules yield information about the state of plastid pigments and their environment. Thus, the absorption of energy in the photosynthetic pigment system opens a window as it were in the unknown building of the photosynthetic apparatus; through this window the light of fluorescence emerges with information on the structure of the apparatus and the processes

going on in it. The power of the method was recently expressed by GOVINDJEE in the following way [1]: "Much of our knowledge of the nature of the two photosystems and of the early events of photosynthesis comes from the study of chlorophyll fluorescence both *in vivo* and *in vitro*". Speaking of the role of pigments in photosynthesis, FRENCH [2] said: "The volume of information about photosynthesis has now become so great that no one person can keep in mind the detailed findings of various laboratories even within a narrow part of the field." "In spite of all the intense effort here reported on many aspects of the mechanism of photosynthesis and on the biophysics of plastid pigments, many basic questions are far from clarified and are, in fact, practically neglected." This statement is still valid, and encourages us to limit the treatment of our subject. Mainly the last few years will be covered in this report and the problems will be selected. For a much broader review GOEDHEER's paper should be consulted [3].

In vivo forms of chlorophylls

A great deal of experimental work has already been done to obtain the absorption spectra of chlorophylls in plant leaves and in algal and chloroplast suspensions. As a result of these experiments it is generally accepted that the red absorption band of *in vivo* chlorophylls is a superposition of the absorptions of several chlorophyll forms [4—8]. These forms have different peak positions and half band widths, generally with small differences. The number and distribution of these forms vary in different photosynthetizing organisms. For the study of the *in vivo* forms of chlorophylls, either Gaussian analysis of the red absorption band [9] or the analysis of the first or second derivative of this band [10] is used. Sometimes the spectra obtained at 77 °K are analysed, since the resolution of the complex red band into its components is much better at low temperatures [9—10]. Fig. 1 shows a typical Gaussian analysis of the photosystem I fraction of Scenedesmus, published by FRENCH (9). FRENCH *et al.* found 4 main forms in different organisms: the forms with absorption peaks at 661.6, 669.6, 677.1 and 683. 7 nm, with half band widths of 11.3, 10.0, 10.3 and 10.8 nm, respectively. The fraction containing photosystem II particles also showed the presence of these 4 forms, but the half band widths were 11.6, 9.8, 9.4 and 9.6, respectively, and the amount of form 684 was greater in fraction I. In addition to these four "universal" forms, especially in fraction I, two more chlorophyll-a forms were claimed with maxima at 693 and 704 nm and half band widths of 13—18 and 14—25 nm in different organisms.

From Gaussian analysis and derivative spectra of suspensions of plant chloroplasts, LITVIN *et al.* [11] assume the existence of even more chlorophyll-a forms. The characteristics of these forms are listed in Table I, taken from [10]. In addition

Table I.

λ (nm)		648	670	670	676	683	686	693	702	712	720
Band width (nm)	293 °K	17	16	16	16	12	12	14	16	16	—
	77 °K	13	12	8.5	9.0	8.0	7.5	11	13	15	—

to the 4 main and 2 accidental forms of FRENCH, 3 more forms, at 686, 712 and 720 nm, are shown here.

Gaussian analysis of the red absorption band of chlorophylls started with two components and a minor third component at the long-wave tail of the band around 700 nm. CEDERSTRAND's analysis of a Chlorella suspension resulted in components

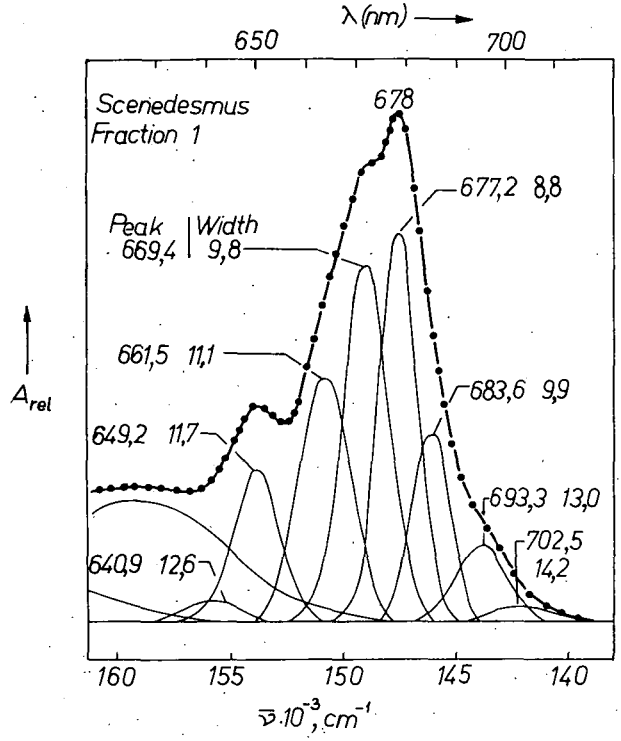

Fig. 1

with 17 nm half width matching very closely the half-width of the red band of chlorophyll-a in solution [12]. In an analysis of the spectrum of a diatom, FRENCH and BROWN [13] compared the results obtained with two, three and four components. They finally accepted the result with four components because the standard error of the approximation was much less in this case.

What is the cause of the appearance of different chlorophyll forms, and what is the origin of these slightly different absorption bands? No generally accepted answer is found to these questions. Chlorophyll-b (640) is assumed to be a monomeric form in the lipid phase, while chlorophyll-a (661), chlorophyll-a (670) and chlorophyll-a (684) are considered to be a monomeric, protein-chlorophyll complex and dimeric absorbing form. To these problems one more should be added: what is the reality of these forms?

There is no doubt that chlorophyll forms with different absorptions of light exist. The half width of the red absorption band *in vivo* is over 30 nm, while that *in vitro* is around 20 nm. Obviously, the *in vivo* red band is a superposition of several absorption bands, originating either from aggregated chlorophylls (the local concentration of the chlorophyll in the chloroplast is as high as $10^{-1}-10^{-2}$ mole/l) or from chlorophyll-protein and chlorophyll-lipoprotein complexes. The specific orientations of these complexes may also play a role in the formation of chlorophylls with different absorptions. There may be some doubt, however, as to the number of the components and the location of the band ascribed to a given component obtained by means of Gaussian analysis.

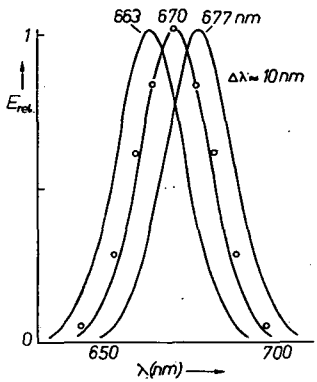

Fig. 2

If the component bands are very near to each other, the resolution of the complex band into its components is more or less arbitrary. Fig. 2 shows three adjacent bands with peaks at 663, 670 and 677 nm as Gaussian distributions with half widths of about 10 nm. The sum of these three distributions (which should be considered as a representation of the measured spectrum) was fitted to the maximum of the band at 670 nm and the composite curve is shown at a few points by open circles. Some of the open circles practically lie on the middle Gaussian curve drawn with a solid line, while some deviate from it, but the deviation is not high. If the band at 670 nm were the measured spectrum, the accuracy of the approximation would seem to be satisfactory. If, however, the sum of three Gaussian distributions with maxima at a distance more than the half width of the components has Gaussian distribution to a good approximation, this is much more true for

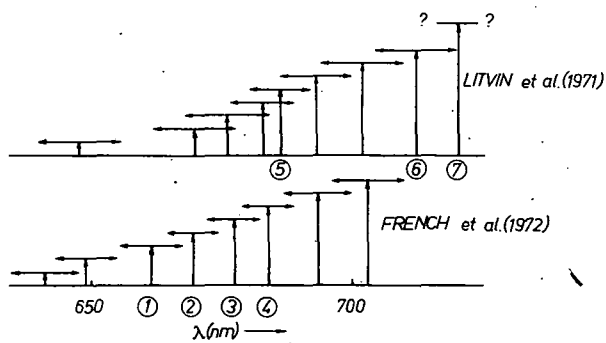

Fig. 3

closer bands. In Fig. 3 a diagram is shown to illustrate how far this point is relevant for the spectral forms assumed by LITVIN *et al.* [11] and FRENCH *et al.* [8].

Sometimes the location of the component bands is found by cooling the samples to the temperature of liquid nitrogen. At this temperature the otherwise smooth

envelope of the band usually exhibits structure and the local maxima are ascribed to the components. In doing so, however, the question arises whether all these local maxima belong to different chlorophyll forms, or whether they belong at least partly to specific low-temperature chlorophyll forms not existing at higher temperatures. Early low-temperature chlorophyll spectra reported and discussed in detail in [3] (p. 1799) show shifts and new bands compared to the spectra obtained at room temperature. The changes are explained by the formation of solvated forms, colloidal forms, the possibility of thermally excitable vibrational states, etc. Whatever the reason of the change of the absorption spectrum on cooling may be in this case, therefore, without additional information the low-temperature spectra cannot be used to predict the location of the bands of the assumed chlorophyll components.

A further difficulty in the Gaussian analysis lies in the fact that the true shape of an absorption band is really *not* Gaussian. This has been recognized by FRENCH *et al.* [15], who approximated the envelope by a mixture of Gaussian and Lorentzian distributions and used computer analysis to try to fit the different components with different ratios of these two distributions. This procedure would result in a better fit, but the physical meaning of an absorption band at 622 nm composed of, for example, 81.0% Gaussian and 19.0% Lorentzian distribution is not quite clear, and in addition it is not clear why another component has another ratio of distribution.

The half widths of the forms given by LITVIN are 12—16 nm at 293 °K (see Table I), and those of the four main forms of FRENCH 10—11 nm. Should these bands belong to chlorophylls in different environments, it is difficult to understand why the half widths are so small. The half widths of chlorophylls have been determined in 40 solvents with different refractive indices, dielectric constants and chemical properties [16]. The range of the half width was 16.6—23.2 nm for chlorophyll-a, far above the 10—11 nm ascribed to the main spectral forms of FRENCH. Much closer to the *in vitro* values are those of the two forms of FRENCH 16.8 and 13.6 nm (with an additional band of 26.3 nm), which were rejected because the standard error of fitting with two forms was higher [13]. It is difficult to assume a specific environment in plants which should lead to a narrow absorption band never found in any *in vitro* environment. If the components were aggregated chlorophylls, the difficulty would be much higher, because the half width of a band of aggregated chlorophyll should be even greater than that of a monomeric form.

The different chlorophyll-a forms play an important part in the migration of electronic excitation energy. The presence of these forms increases the frequency of the transfer of energy to the reaction center. According to SEELY [17], the frequency of transfer is 3—5 times higher in a heterogeneous photosynthetic unit (with three chlorophyll-a forms absorbing at 670, 678 and 686 nm) than in a homegoneous unit. The same conclusion was obtained by BORISOV *et al.* [18], with calculations extending to different forms of bacteriochlorophyll. It would therefore be very important to find a satisfactory way of obtaining these forms.

In studying the multiplicity of chlorophyll-a forms attention also has to be paid to the sieve effect. In an earlier work [19] the peaks of the two components were found to be shifted from 668 to 670 nm and from 683 to 680 nm if the sieve effect appearing in a Chlorella suspension were removed.

The facts mentioned above lead to the conclusion that absorption spectra

taken without attention to possible artefacts (light scattering, sieve effect, etc. [20]) cannot be satisfactorily used for Gaussian analysis, and Gaussian analysis alone cannot lead to acceptable components. Pure chlorophyll-a in 80% acetone solution with no chlorophyll-b, had a "hidden" component absorbing at 650 nm, obviously a product of the Gaussian analysis [15]. In addition, several chlorophyll-a forms with very near bands would introduce an error into the Gaussian analysis because of the strongly overlapping vibrational bands preceding the main red band.

The analysis of fluorescence data can contribute to a better understanding of the multiplicity of chlorophyll-a forms.

In earlier papers *in vivo* fluorescence data involving different chlorophyll-a forms were succesfully analysed. The red drop of the quantum yield of fluorescence of algae [21], the red drop of the fluorescence of sonicated algae [22], the change of the fluorescence spectrum in ageing bean leaves [23], and the effects of "blue" and "red" light on the development of the pigment system of Sinapis alba [24] could be interpreted in terms of two main forms of chlorophyll-a. The excitation spectra of fluorescence in Chlorella showed two peaks at around 670 and 680 nm [20], as would be expected after [19]. These findings and the difficulties enumerated in connection with the Gaussian analysis give us the impression that two or three main forms could more easily be accepted. This statement, however, does not exclude the existence of several minor components in between or toward the shorter or longer waves. As a result of Gaussian analysis, these components in smaller amounts would, however, have reasonably greater band widths.

According to PAILLOTIN [25] the movement of excitons is not a Markoffian random walk, since the "jump over time" t_j, i.e. the time needed for the excition to make one jump, is not much less than the "time between jumps", t_D. For the estimation of these times the 450 cm^{-1} ($\approx$21 nm) half width of the chlorophyll-a red absorption band *in vivo* was accepted. With this value $t_D=0.07$ sec and $t_j=0.03$ sec. With band widths of 10—12 nm, the values are: $t_D=0.035$ sec and $t_j=0.005$. In the latter case $t_j \ll t_D$ and the condition for a Markoffian process is fulfilled. From the point of view of the microscopic properties of energy transport, therefore, the knowledge of *in vivo* band widths is very important.

Migration of electronic excitation energy in detergent (micellar) systems

The absorption of light in the photosynthetic pigment system is followed by a complex process of energy migration, the details of which are not fully known. During this process, the absorbed energy reaches special chlorophyll-a molecules performing the conversion into chemical energy in the reaction center of a "photosynthetic" unit containing about 300 chlorophyll molecules, according to the unicentral model of photosynthesis [26]. The migration of energy is part of the primary process of photosynthesis which also includes the absorption of light by the different forms of chlorophylls (and accessory pigments) mentioned above. The primary process is followed by a secondary one in the reaction center and in its environment, involving photochemical, biochemical and electron transport phenomena [27].

The knowledge of the physical processes of photosynthesis is difficult because in living systems, even the chemically identical molecules are present in different physical states (*e.g.* as aggregates). In addition, the pigments may be bound to the

components of chloroplast, while the living systems are very sensitive to light, heat and other experimental conditions, and in many cases the composition of the pigment system and the pigment concentrations are unknown.

The difficulties can be eliminated, at least partly, if the processes are studied in models approximating the structure and properties of the photosynthetic apparatus. Among the possible models there is the often applied detergent micellar system [28]. Micelle formation occurs if amphoteric compounds (*e.g.* sodium-lauryl-sulfate, SLS) are dissolved in water. The ions formed in the detergent solution will be ordered into spherical or lamellar structures to decrease the surface energy. The micellarization starts at a well-defined concentration, the „critical micelle concentration" (CMC), characteristic of the detergent [29].

For the investigations reported here SLS was applied as detergent. During micellization 80—100 lauryl sulfate ions are connected into a micelle. The formation of micelles is comparatively easy to observe, since several physical properties of the solution (electric conductivity, absorption of light, etc.) change when the micelles appear in the solution. As Fig. 4 shows, the structure of SLS-micelles is suitable

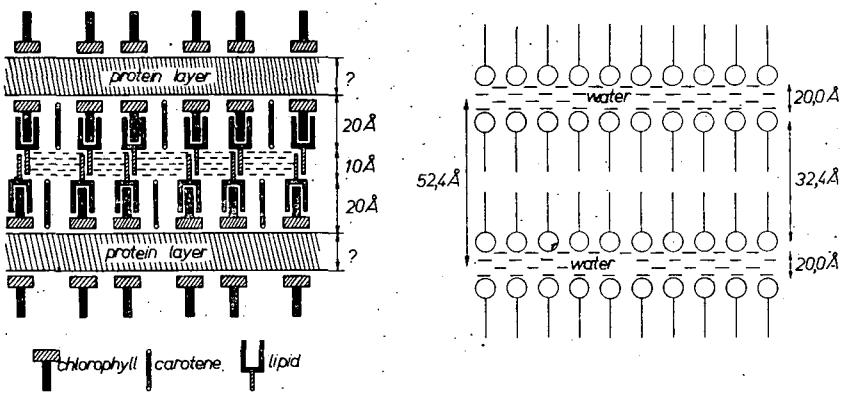

Fig. 4

for modeling the primary process of photosynthesis. The ultrastructure of the chloroplast and the structure of a micelle formed by LS^--ions and water have been shown by X-ray diffraction [28] to be very similar, even the sizes being comparable.

In our studies, thionine (Th), methylene blue (MB) and rhodamine 6G (Rhod 6G) dyes, or suitable pairs of these, were incorporated into the micelles. These dyes are advantageously applied in detergent solutions because: 1. they dissolve readily in water and the solution is well fluorescent, 2. the photosynthetically important spectral region is practically covered by their absorption and fluorescence spectra. Consequently, these dyes are conveniently used to replace the photosynthetic pigment forms in models. At suitable dye concentrations the order of the distance of the dyestuff ions incorporated in the micelles is comparable with the average distance of the pigments in the chloroplast [30].

In two-component systems the transfer of excitation energy can be studied either by measuring the decrease in the intensity of fluorescence of the donor dye (the quenching of the fluorescence,) or by determining the increase in the inten-

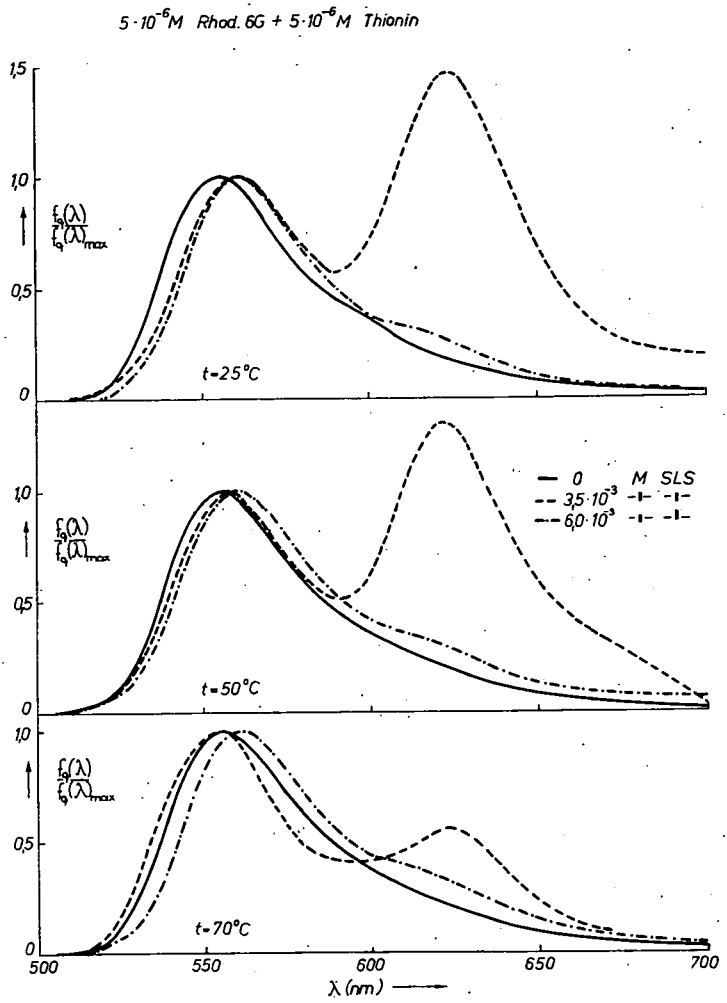

Fig. 5

sity of the fluorescence of the acceptor dye (sensitization of fluorescence). Rhod 6G+Th, and Th+MB micellar solutions were studied at different temperatures from the point of view of energy transfer. The fluorescence spectra of $5 \cdot 10^{-6}$ M equimolar mixtures of Rhod 6G+Th at 0, 3.5 and $6.0 \cdot 10^{-3}$ M detergent concentracions are shown in Fig. 5. A high increase is seen in the emission of Th. The increase is less at higher temperatures, but it is present even at the highest temperature studied. At a detergent concentration of $6 \cdot 10^{-3}$ M no increase of the emission of Th is observed.

In Fig. 6 the fluorescence spectra of $5 \cdot 10^{-6}$ M equimolar mixtures of Th and MB are plotted as a function of the detergent concentration at room temperature [31].

In water solution the intensity of fluorescence of Th is higher than that of MB. If detergent is introduced into the solution, however, the relative intensity of the fluorescence of MB increases with the increase of the detergent concentration. In $4 \cdot 10^{-3}$ M SLS solution the intensity of fluorescence of MB is five times higher than that of Th. A further increase in the detergent concentration leads to a decrease in the relative fluorescence intensity of MB.

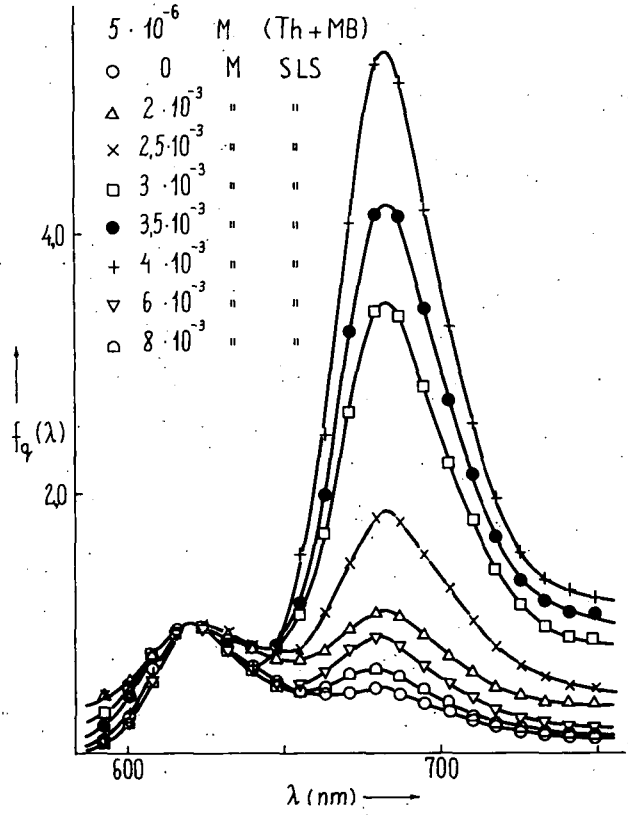

Fig. 6

From Figs. 5—6 the following conclusion can be drawn. In the systems studied, the probability of energy transfer is optimum at room temperature and at a detergent concentration corresponding to the CMC. If the concentration of detergent is higher, due to the increase of the number of micelles, the average distance between adjacent dye molecules increases, and therefore the probability of energy transfer is less. If the temperature of the system is higher, due to the decrease of the number of micelles, the distance between the dye molecules becomes smaller than the optimum, and therefore the probability of energy transfer is less again.

Fig. 7 shows the relative fluorescence intensities of $5 \cdot 10^{-6}$ M Th solution at 25 and 50 °C. The changes in fluorescence intensity were determined from the fluorescence

excitation spectra of solutions containing Th only and solutions containing a mixture of Th and MB at the same wavelength [32]. According to Fig. 7 the decrease of the intensity of Th fluorescence depends on the detergent concentration and on the temperature, revealing the increase of energy transfer. Typical quenching curves are obtained as are expected in the quenching process of fluorescence of Th by an energy transfer to MB.

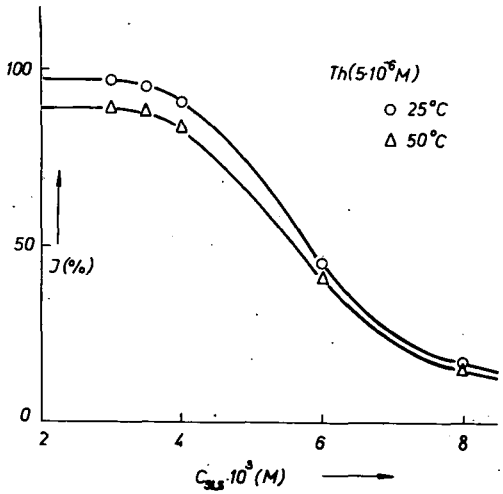

Fig. 7

If the number of micelles and the concentration of the dye in a solution are known, the number of dye molecules in a micelle and — assuming random distribution—the average distance between adjacent dye molecules can be calculated [33]. Calculations were carried out for mixtures of $5 \cdot 10^{-6}$ M Rhod 6G and Th with different concentrations, with the assumption that all of the dye molecules are incorporated into the micelles. The calculations showed an optimum for both the number of dyestuff molecules in a micelle and the distance of the dye molecules from the point of view of energy transfer. The highest effectivity of transfer is found in systems where 12—20 dyestuff molecules are included in a micelle and their average distance is 24—40 Å (Table II). If the distance between the adjacent pigment molecules in the micelles is higher than the optimum, the effectivity of transfer is smaller.

These calculations showed that with the increase of the number of acceptor molecules (Rhod 6G) the optimum is shifted towards higher detergent concentrations.

Table II.

C_{SLS} / C_{dye}	$3.0 \cdot 10^{-3}$ M		$3.5 \cdot 10^{-3}$ M		$4.0 \cdot 10^{-3}$ M		$6.0 \cdot 10^{-3}$ M	
	M	*d*	M	*d*	M	*d*	M	*d*
Rhod 6G	13	38	6	83	4	125	2—3	200
$+2 \cdot 10^{-6}$ M Th	18	28	8	62	6	83	3—4	150
$+5 \cdot 10^{-6}$ M Th	26	19	12	41	8	62	5	100
$+1 \cdot 10^{-5}$ M Th	38	13	17	30	13	38	7	71
$+2 \cdot 10^{-5}$ M Th	64	7—8	29	17	21	24	12	41

M = number of dye molecules within a micelle,
d = distance of dye molecules within a micella, in Å.
The numbers belonging to the optimum transfer are underlined (see text).

This is probably explained by a higher probability of transfer from Th due to the greater number of acceptors.

After the properties of organic dyestuff—detergent systems had been clarified, the study of photosynthetic pigment—detergent systems (especially the migration of energy) was carried out. In this model, since plant pigments are present, the *in vivo* properties of the chloroplast are much better approximated. Because of the presence of the LS^- ion, the chlorophylls could not be incorporated into SLS micellar solutions; triton-X100 detergent, widely used in photochemical investigations of chlorophylls, was applied. The optical properties of chlorophyll—triton—X100 solutions prepared according to the usual methods (*e.g.* [34, 35]), were found to be unstable, especially at higher pigment concentrations. A method of preparation of pigment detergent solutions with stable spectral properties was therefore elaborated [37].

The absorption and fluorescence (fluorescence spectrum, fluorescence excitation spectrum, degree of polarization and yield of fluorescence) of chlorophyll-b and

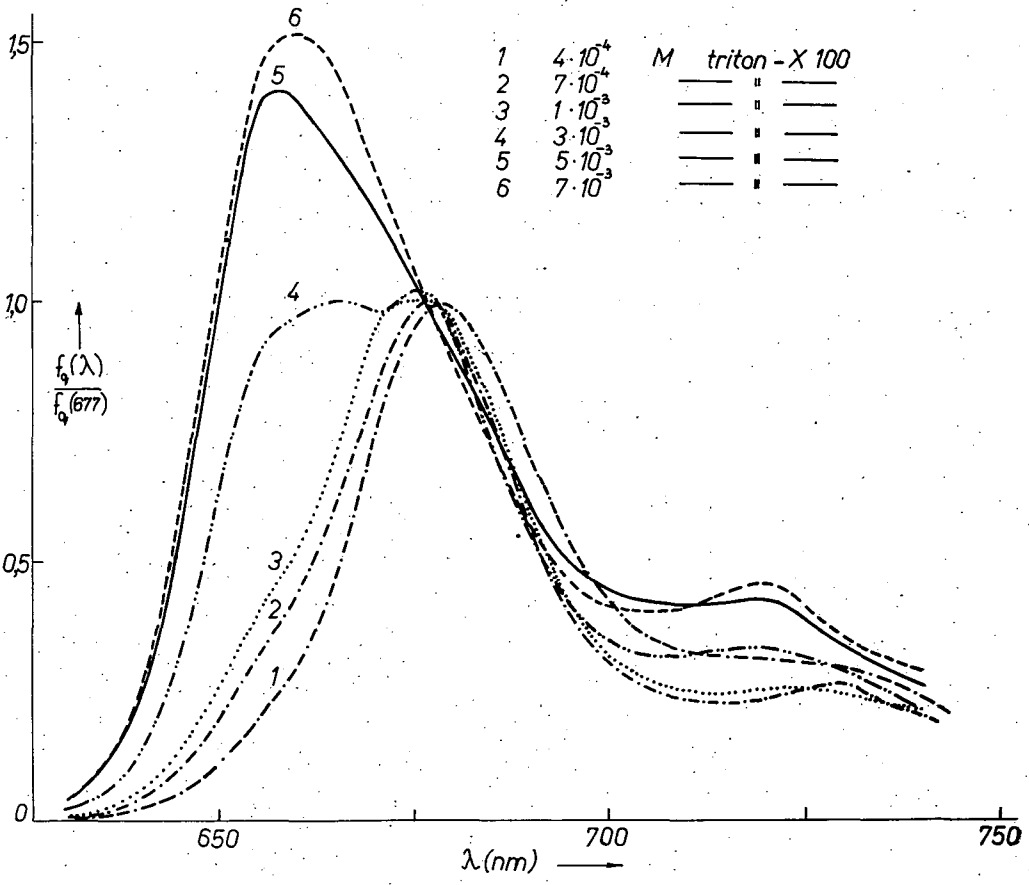

Fig. 8

chlorophyll-a mixtures and of mixtures of lutein and chlorophyll-a were measured in detergent solutions as functions of the pigment concentration and the temperature. From the results of these measurements conclusions were drawn about the transfer of energy from chlorophyll-b and lutein to chlorophyll-a.

The relative fluorescence spectra of $6 \cdot 10^{-6}$ M equimolar mixtures of chlorophyll-a and chlorophyll-b are shown in Fig. 8 with excitation at the absorption maximum of chlorophyll-b (466 nm), where the absorption of chlorophyll-a is negligible. The efficiency of energy transfer depends highly on the detergent concentration. Based upon the quenching of the fluorescence of the acceptor (chlorophyll-a) the effectivity of energy transfer from chlorophyll-b to chlorophyll-a was calculated [38]. In acetone solution the effectivity was 0.04, and in micellar solutions with triton concentrations above CMC 0.87; 0.78; 0.62; 0.38 and 0.15, according to the concentrations shown in Fig. 8. These data show a very effective transfer of energy in chlorophyll detergent systems even at small pigment concentrations, and especially at low detergent concentrations.

The absorption spectrum of an equimolar lutein—chlorophyll mixture in $3 \cdot 10^{-4}$ M triton, and the fluorescence excitation spectra of chlorophyll-a and the mixture observed at the maximum of fluorescence of chlorophyll-a (680 nm) are shown in Fig. 9. The effectivity of the energy transfer from lutein to chlorophyll-a

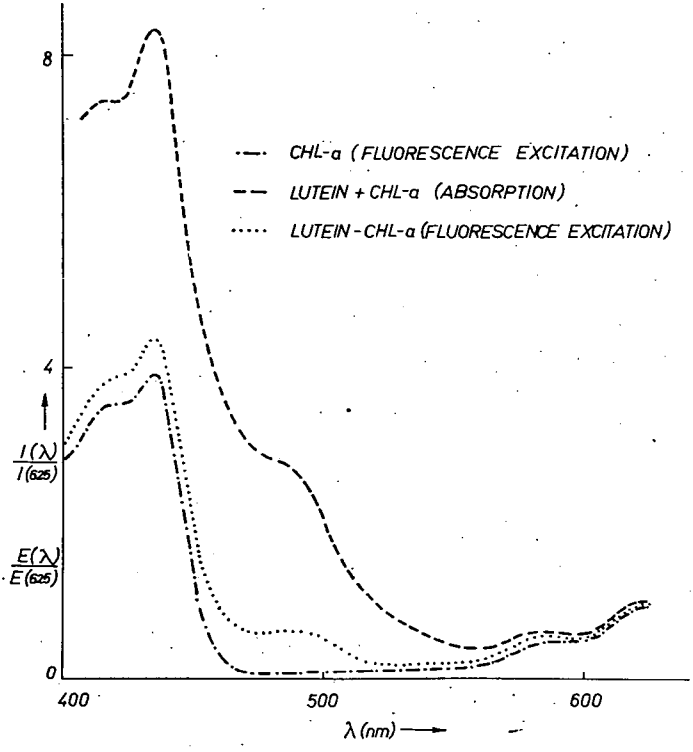

Fig. 9

was calculated according to [39]. The maximum effectivity was also found at small detergent concentrations, but it never exceeded 0.25.

We assume that the role of the detergent in the transfer of energy is twofold: the detergent micelles ensure a high local concentration of pigments, promoting the transfer, and they render possible an oriented incorporation of the pigment into a structure, also favouring transfer.

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МОЛЕКУЛЯРНАЯ ФЛУОРЕСЦЕНЦИЯ И ФОТОСИНТЕЗ

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Обсуждается обоснованность существования форм хлорофилла с полушириной полосы поглощения 10—12 нм полученных методом гауссовского анализа красной полосы поглощения зеленых растений. Главные трудности связанные существованием таких форм и разложением спектров следующие:

1. Поскольку гауссовские компоненты расположены очень близко друг к другу разложение полосы поглощения является более менее самовольным.

2. При низких температур расположение компонентов более четко видно, однако могут появлятся специфические низкотемпературные формы несуществующие при комнатной температуре.

3. Настоящий вид полосы не является гауссовским.

4. Поскольку полосы различных форм хлорофилла расположены очень близко, перекрывающие колебательные полосы могут приводить к ошибкам в анализе.

5. В растворах полуширины красных полос без исключения составляет 16—23 нм, намного больше значения 10—12 нм данного для нескольких in vivo форм.

На основе данных флуоресценции предпологается признать двух-трех главных и несколько побочных (низкие концентрации) форм обладающихся реальной полушириной полосы поглощения.

Мицеллярные растворы детергента (натрий-лаурилсульфата) содержащие тионин, метиленовый голубой, родамин 6Ж и их смесей исследовалось как модельная система фотосинтетической единицы. Спектры поглощения и флуоресценции показывают наличие как мономерной и агрегированных форм, так и комплекса красителя-детергента выше названных красителей, а также перенос возбуждающей энергии в этих системах. Эффективность передачи энергии достигает максимум если в мицеллах находится в среднем 12—20 молекул красителей и среднее расстояние между которыми составляет 25—40 Å.

Фотосинтетические пигменты растений (хлорофилл-а, хлорофилл-в и лютеин) можно встроить в мицеллы тритона-X100. Обсуждается миграции возбуждащей энергии в такой модельной системе с хлорофилла-в на хлорофилл-а и с лютеина на хлорофилл-а.